CLINICAL STUDY

The effects of thyroid hormones on circulating markers of cell-mediated immune response, as studied in patients with differentiated thyroid carcinoma before and during thyroxine withdrawal

José I Botella-Carretero[1], Aranzazu Prados[2], Luis Manzano[3], María T Montero[4], Luis Escribano[2], José Sancho[1] and Héctor F Escobar-Morreale[1]

Departments of [1]Endocrinology, [2]Haematology–Mastocytosis Unit, [3]Internal Medicine and [4]Biochemistry-Research, Hospital Ramón y Cajal, Madrid, Spain

(Correspondence should be addressed to Héctor F Escobar-Morreale, Department of Endocrinology, Hospital Ramón y Cajal, Carretera de Colmenar Km. 9.100, 28 034 Madrid, Spain; Email: hescobarm.hrc@salud.madrid.org)

Abstract

Objective: To address the influence of thyroid hormones on circulating markers of cell-mediated immune response in an *in vivo* human model.

Subjects and design: Twenty-two patients with stage I differentiated thyroid carcinoma were studied on the last day of thyroxine suppressive treatment, 4–7 days after withdrawal, and the day before whole body scanning. Three patients were excluded because of residual disease. Twenty euthyroid individuals served as controls. Serum thyrotrophin and thyroid hormones were measured by an immunometric assay, circulating cytokines by enzyme-linked immuno-sorbent assay and lymphoid populations by flow cytometry.

Results: Thyroid function in patients changed from subclinical or mild hyperthyroidism at the first visit, to a situation of normal circulating levels of free thyroxine and triiodothyronine at the second, ending in a state of overt hypothyroidism. Thyroxine suppressive treatment in patients increased serum interleukin-18 concentrations (IL-18, mean±s.d., 280±122 vs 183±106 pg/ml, F = 3.192, $P = 0.029$), soluble interleukin-2 receptor levels (sIL-2R, 4368±1480 vs 2564±846 pg/ml, F = 21.324, $P < 0.001$), and the percentage of natural killer (NK) cells in peripheral blood (15.9±8.6 vs 10.5±3.6%, F = 4.977, $P = 0.004$) compared with controls. After thyroxine withdrawal, serum levels of IL-18, sIL-2R and the percentage of NK cells decreased progressively.

Conclusion: Our present results suggest that thyroid hormones modulate the cell-mediated immune response in humans.

European Journal of Endocrinology **153** 223–230

Introduction

Modulation of the immune response by thyroid hormones has been well recognised for years. Although thyroid hormones are required for normal B lymphocyte development (1) and humoral immune response (2), it is unclear whether they play a significant role in T lymphocyte development and cell-mediated immunity (1).

Thyroidectomy in animals results in depressed humoral and cell-mediated immune responses (3). However, the study of the influence of exogenous thyroid hormones on the immune response yielded conflicting results (2). Many studies in humans conducted *in vivo* included patients with autoimmune disorders, or patients treated with thionamides, and these factors may have influenced the immune response in addition to the direct effects of thyroid hormones, thus acting as confounding factors (4, 5).

At present, there is increasing evidence that the cell-mediated immune response, directed towards virally infected cells and tumour cells, is mainly mediated by natural killer (NK) cells, cytolytic T lymphocytes, and activated macrophages (6–8). Macrophages and NK cells are considered innate immunity effectors, yet the adaptive immune response that occurs after T lymphocyte activation is also important for the functional regulation and proliferation of NK cells and macrophages (9). Interleukin (IL)-18 and IL-12 are secreted by macrophages, and induce the production of interferon-γ (IFN-γ) by T lymphocytes and NK cells (10, 11). Therefore, these cytokines are important effectors of the adaptive immune response directed towards the activation and proliferation of macrophages and NK cells.

In this process, T lymphocytes release IL-2, which acts through autocrine and paracrine mechanisms to stimulate the functional activity and growth of T lymphocytes and NK cells (9). The high affinity IL-2 receptor is a cell membrane heterodimer composed of α-, β- and γ-subunits. T lymphocyte stimulation is followed by shedding of the α-subunit (also known as CD25), which may be measured in serum. This fraction is termed soluble interleukin-2 receptor (sIL-2R), and is considered a specific serum marker of T cell proliferation and activation (9).

In the present study we studied several markers of cell-mediated immune response in patients with a history of differentiated thyroid carcinoma without any current evidence of residual disease. By evaluating these markers when on chronic thyroxine suppressive therapy, and after thyroxine withdrawal, we estimated the direct influence of thyroid hormones on cell-mediated immunity, avoiding the possible effects of autoimmunity or residual cancer that might have acted as confounding factors in previous studies.

Subjects and methods

Twenty-two women with differentiated thyroid carcinoma, referred for a routine whole body scan during follow-up after initial total thyroidectomy and ^{131}I ablation, were recruited prospectively. Patients were studied during thyroxine withdrawal at three time points: the last day on thyroxine at their usual thyrotrophin (TSH)-suppressive doses, 4–7 days after withdrawal (12), and the day before ^{131}I whole body scanning. Thyroid function in these patients was expected to change from subclinical or mild hyperthyroidism at the first visit, to a situation of normal circulating levels of free thyroxine and triiodothyronine at the second, ending in a state of overt hypothyroidism at the last visit.

The indication for whole body scanning after thyroxine withdrawal (recombinant human TSH was not available in Spain at the time of the study), as well as the degree of suppression of endogenous TSH secretion, and the doses of thyroxine used during follow-up, were decided by the physicians referring these patients, and were not influenced by any of the authors of the study. Two patients were excluded from the study because their baseline TSH levels were not suppressed. The remaining patients had been taking TSH-suppressive doses of thyroxine (mean±S.D.: 169±24 μg per day) for 44±53 months (range 6 – 249 months), after total thyroidectomy and ^{131}I ablation of thyroid remnants. At that time, all the patients had stage I differentiated thyroid carcinoma (14 papillary, 5 follicular and one mixed papillary-follicular).

The age of the patients was 43±14 years at recruitment, and before the present whole body scanning, 17 of the 20 patients had no evidence of thyroid remnants because serum thyroglobulin levels were undetectable. The remaining three patients were excluded from the analysis, two because of detectable thyroglobulin levels and because cervical remnants on whole body scanning were found, and one because of pulmonary metastases. The mean±S.D. total cumulative ^{131}I dose received before the study by the seventeen patients finally analysed was 132±87 mCi, and none of these patients had received any ^{131}I dose for the previous six months before sampling.

Twenty healthy female volunteers, matched for age (43±15 years) and without thyroid disease, served as controls. They were not taking any drug known to influence thyroid function. Preliminary data regarding quality of life, psychometric functionality and cardiovascular aspects of most of the patients and controls have been reported previously (13, 14).

The ethics committee of the Hospital Ramón y Cajal approved the study, and written informed consent was obtained from all the participants.

Study protocol and analytical procedures

Patients and controls reported early in the morning after a 12-h fast. Patients were advised to take their usual thyroxine dose just after waking up, before reporting to the hospital on the day of the first visit, and medication was withdrawn thereafter. Evaluation was repeated in the patients after 4 to 7 days (second visit, mean±S.D.: 5.4±1.1 days after thyroxine withdrawal), and the day before whole body scanning was performed (third visit: 30.6±4.4 days after thyroxine withdrawal). Controls were evaluated only at one time point.

Serum samples were obtained for determination of TSH, free thyroxine (FT4) and free triiodothyronine (FT3) using commercial immunochemiluminescent assays (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA). The mean coefficients of variation were below 10% for all these assays. The normal ranges were 0.4 – 4.0 μU/ml for serum TSH, 0.8 – 1.9 ng/dl for FT4, and 1.8 – 4.2 pg/ml for FT3 as reported by the Central Laboratory of the Hospital Ramón y Cajal.

Serum cytokine concentrations were measured in duplicate by enzyme-linked immuno-sorbent assay (ELISA). For IL-6, which is predominantly implicated in systemic inflammation (15), a high sensitivity commercial kit was employed (Diaclone, Besançon, France) after adjusting the standard curve to yield a sensitivity of 0.4 pg/ml. Serum sIL-2R levels were measured using a commercial kit (Diaclone) with a sensitivity of 8 pg/ml. Serum IFN-γ and IL-12 were determined by commercial ELISA kits (Diaclone) with a sensitivity of 5 pg/ml and 20 pg/ml respectively. Finally, serum IL-18 was measured by ELISA (MBL, Naka-ku, Nagoya, Japan), after adjusting the standard curve to reach a sensitivity of 12.8 pg/ml. The mean coefficients of variation were below 10% for all these assays.

Lymphoid subpopulations were studied in a subgroup of 13 patients and 16 controls. This subgroup was representative of the whole population of patients and controls in terms of age (45 ± 12 years and 41 ± 15 years respectively), thyroxine dose ($162\pm22\,\mu$g per day) and ^{131}I cumulative doses (139 ± 98 mCi) in patients.

Total blood samples were analysed for white cells count. For the study of lymphoid subpopulations and activity markers, the following monoclonal antibody panels were employed: CD8-FITC/CD19-FITC/CD3-PE/CD56-PE and CD4-PerCP (Lymphogram, Vitro S.A. Madrid, Spain), and CD3-FITC/CD4-PE/HLA-DR PerCP/CD25-APC, and CD3- FITC/CD4-PE/CD8-PerCP/CD69-APC (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry (FACSort, Becton Dickinson) was performed after incubation, lysis and washing of samples, and then analysed by specific software (Cell-Quest and Paint-A-Gate, Becton Dickinson). Lymphoid subpopulations were expressed as percentage of the total lymphocyte count, which, in turn, was expressed as percentage of the total leukocyte count.

Statistical analysis

Data are expressed as means$\pm$S.D. unless otherwise stated. The normal distribution of the variables was analysed using the Kolmogorov-Smirnov test. Logarithm or square root transformations were applied to ensure normal distribution whenever possible. The values of the patients at the three visits were compared by repeated measures analysis of variance, or Friedman analysis of variance by ranks, as appropriate. After Friedman analysis, comparisons between visits 1, 2 and 3 were performed using repeated Wilcoxon signed rank tests applying the Bonferroni correction to the level of significance.

The comparisons of the values of the patients at each visit with the controls were performed by one-way analysis of variance followed by Dunnet's test, or by Kruskall-Wallis one-way analysis of variance by ranks followed by Mann-Whitney tests, depending on the distribution of the variables. After a significant Kruskall-Wallis test was obtained, the identification of the particular visit, or visits, which were different compared with the controls, was made using separate Mann-Whitney tests. Because no comparisons were made between visits, no correction was applied to the level of significance. An α value of 0.05 was chosen as the level of statistical significance with the exceptions described above. Statistical analyses were performed using SPSS for Macintosh, version 10 (SPSS Inc., Chicago, IL, USA).

Results

Serum thyroid hormone concentrations (Fig. 1)

As expected from chronic treatment with supraphysiological doses of thyroxine, the mean serum thyroid hor-mone levels were in the mild hyperthyroid range at visit 1 (14 of 17 patients had increased FT4 levels, whereas all the patients had suppressed TSH levels, and only two had increased FT3 concentrations) (Fig. 1).

At visit 2, mean TSH levels were below the normal range, whereas mean serum FT4 and FT3 were within the normal range. Fourteen of 17 patients still had decreased TSH levels, but their FT4 and FT3 levels were within the normal range except in one patient who still had minimally increased FT4 levels. When considering patients as a group, at visit 2 these subjects presented with lower mean TSH and FT3 levels compared with euthyroid controls (Fig. 1).

At visit 3, all the patients presented with increased serum TSH and low serum FT4 and FT3 levels, with the exception of two patients who had FT3 levels in the lower limit of the normal range. When considering patients as a group, mean serum TSH was increased, and mean FT4 and FT3 levels were decreased, compared with euthyroid controls and with the normal range established in the Central Laboratory of the Hospital Ramón y Cajal (Fig. 1).

Serum cytokines and lymphoid populations in patients on TSH-suppressive thyroxine treatment, and compared with euthyroid controls (Table 1 and Fig. 2)

Patients receiving TSH-suppressive doses of thyroxine presented increased levels of serum IL-18 and sIL-2R compared with controls, suggesting a potential stimulation of the adaptive immune response. On the other hand, serum IL-6 levels of patients on TSH-suppressive thyroxine treatment were not different from those of controls, suggesting that a stimulation of systemic inflammation was not present (Fig. 2).

In conceptual agreement with this cytokine pattern, the percentage of peripheral blood NK cells was significantly increased in patients when on TSH-suppressive thyroxine treatment, compared with controls. However, the percentage of CD4 + T lymphocytes in patients at this time was lower compared with controls. No differences among patients when on TSH-suppressive therapy and healthy controls were found in any of the other lymphoid populations or immune markers studied here (Table 1).

Serum cytokines and lymphoid populations during thyroxine withdrawal, and compared with euthyroid controls (Table 1 and Fig. 2)

Thyroxine withdrawal was associated with a progressive decrease in sIL-2R levels, which were low when patients were severely hypothyroid compared with previous visits, and also compared with controls (Fig. 2). Similarly, serum IL-18 levels in patients decreased in parallel with serum thyroid hormones, and were low during overt hypothyroidism compared with previous
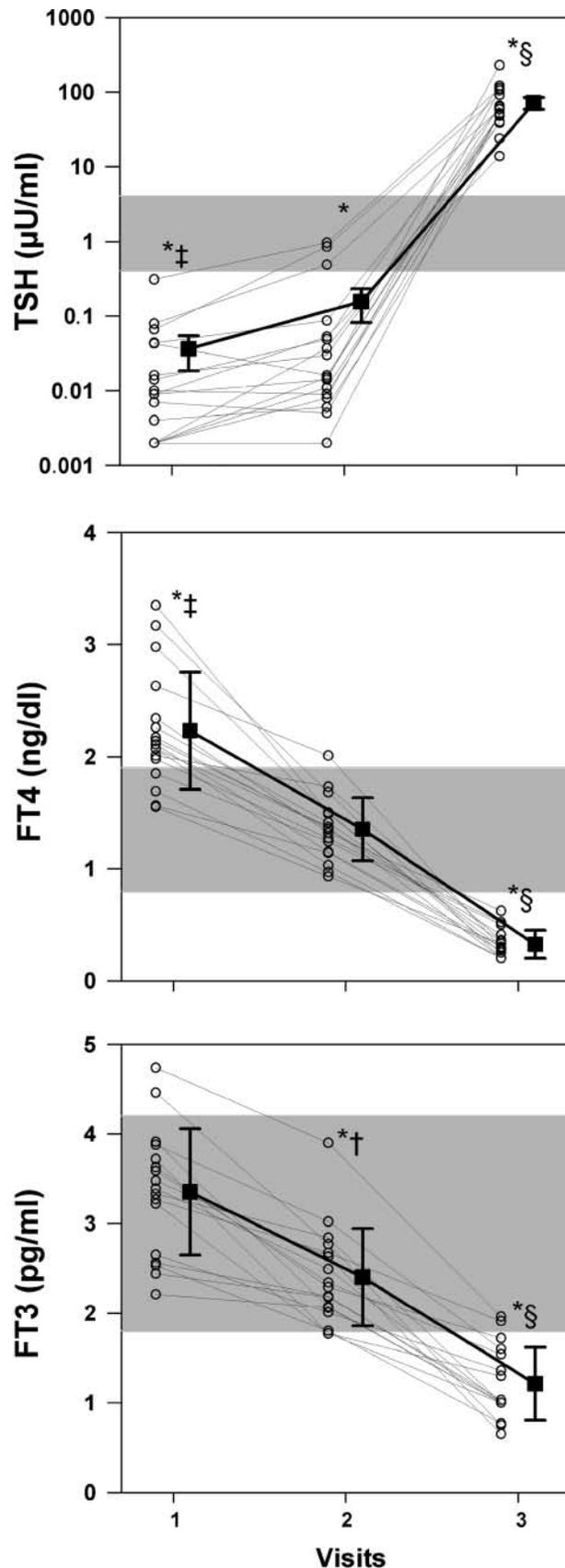
visits, but not compared with controls (Fig. 2). Finally, serum IL-6 levels remained unchanged during thyroxine withdrawal (Fig. 2), whereas serum IL-12 and IFN-γ were undetectable in both patients and controls throughout the study.

The percentage of NK cells in peripheral blood decreased during thyroxine withdrawal, compared with that found when patients were on TSH-suppressive doses of thyroxine (Table 1). This decrease in the percentage of NK cells was evident at visit 2, when patients presented FT4 and FT3 levels in the normal range, and was even more important at visit 3, when patients were overtly hypothyroid. However, when compared with controls, no differences were found in the percentages of NK cells when patients presented FT4 and FT3 levels in the normal range or when they were hypothyroid.

Discussion

Considering that the changes in cytokine levels and lymphocytes observed in our patients paralleled the changes in circulating thyroid hormone concentrations, our present results suggest that thyroid hormones directly modulate circulating markers of cell-mediated immune response *in vivo* in humans.

On the one hand, TSH-suppressive thyroxine treatment increased several markers of cell-mediated immune response, including serum IL-18 and sIL-2R levels, and the percentage of NK cells in peripheral blood. On the other hand, these immune changes are apparently dependent on serum thyroid hormones, as the predominant cell-mediated immune response observed in our patients during TSH-suppressive therapy blunted shortly after thyroxine withdrawal, when serum IL-18 levels and the percentage of NK cells returned to levels similar to those of controls. Moreover, when patients were overtly hypothyroid, sIL-2R levels decreased even below those of healthy euthyroid controls.

These results provide original and intriguing experimental evidence suggesting that TSH-suppressive therapy is associated with an expansion of NK cells,

Figure 1 Thyroid hormone function in patients with differentiated thyroid carcinoma on suppressive thyroxine treatment and during thyroxine withdrawal, and also compared with euthyroid controls. Data are represented as means±S.D., and the dot scattergram shows the individual data with lines connecting them. The shaded areas represent the normal ranges for serum thyroid hormone levels as reported by the Central Laboratory of the Hospital Ramón y Cajal. Statistically significant differences between visits 1, 2 and 3 were observed in serum TSH, FT4 and FT3 levels, with $P < 0.05$ or less for all the comparisons. *$P < 0.05$ or less compared with the values from the euthyroid control group, which were TSH = 1.87±0.88 μU/ml, FT4 = 1.29±0.13 ng/dl and FT3 = 3.38±0.77 pg/ml; †$P < 0.05$ or less compared with visit 1; ‡$P < 0.05$ or less compared with visit 2; §$P < 0.05$ or less compared with both visits 1 and 2.

Table 1 Lymphoid subpopulations in the subgroup of patients with differentiated thyroid carcinoma and euthyroid controls studied by flow cytometry.

	Patients ($n = 13$)			
	Visit 1	Visit 2	Visit 3	**Controls** ($n = 16$)
Serum thyroid hormone levels				
TSH(μU/ml)	0.04±0.08*‡	0.16±0.31*	78.02±55.65*§	1.75±0.81
FT4 (ng/dl)	2.18±0.47*‡	1.33±0.30	0.32±0.14*§	1.31±0.12
FT3 (pg/ml)	3.15±0.71	2.42±0.56*†	1.20±0.40*§	3.51±0.78
Lymphoid populations				
Total leukocytes (n/mm³)	6,551±1,860	6,011±1,717	6,280±1,795	6,067±1,367
Total lymphocytes (%)	29.63±6.11	31.43±8.18	31.51±7.55	31.56±5.70
NK (CD56 + CD3 −) cells (%)	15.91±8.64*	9.84±4.10†	7.97±4.66†	10.47±3.61
T(CD3 +) lymphocytes (%)	70.03±10.91*	73.69±6.02	79.02±7.51	76.76±8.98
CD4 + lymphocytes (%)	38.81±7.63*	42.49±6.83	43.82±7.65	48.34±7.85
CD4 + 25 + lymphocytes (%)	30.79±15.46	30.49±10.05	40.10±21.66	33.10±23.60
CD4 + 69 + lymphocytes (%)	12.13±28.76	8.92±27.40	4.11±9.95	6.05±16.82
CD4 + DR + lymphocytes (%)	2.42±1.43	2.40±1.67	2.13±1.52	8.07±22.82
CD8 + lymphocytes (%)	22.98±5.51	22.23±6.80	27.08±5.02	22.68±5.89
CD8 + 25 + lymphocytes (%)	10.18±7.42	6.53±5.28	21.44±35.46	16.11±32.90
CD8 + 69 + lymphocytes (%)	7.19±9.51	4.69±3.81	3.87±5.47	3.12±2.18
CD8 + DR + lymphocytes (%)	4.61±3.15	4.33±2.53	6.69±7.69	7.53±8.43
CD4 + 8 + lymphocytes (%)	1.16±1.22	1.55±1.67	1.52±2.06	1.11±0.96
CD4 − 8 − lymphocytes (%)	7.08±5.92	7.42±6.89	6.78±7.48	5.74±3.18
B (CD19 +) lymphocytes (%)	13.97±3.94	15.69±4.90	15.88±4.79	12.88±5.27

*$P < 0.05$ or less when compared with euthyroid controls; †$P < 0.05$ or less when compared with visit 1; ‡$P < 0.05$ or less when compared with visit 2; §$P < 0.05$ or less when compared with visit 1 and 2.

which represent the first line of cell-mediated defence against tumour cells (7). Moreover, considering that NK and cytolytic T-cells activation and proliferation are induced by several cytokines such as IL-2 and IL-18 (10, 16), the increase in serum sIL-2R and IL-18 levels when patients were on TSH-suppressive therapy may be related to the increase in the percentage of NK cells in peripheral blood. As anti-tumour effects have recently been shown to be enhanced by immunotherapy with IL-18, which in turn activates NK cells and other cytolytic T lymphocytes (17–19), our present results, if confirmed by larger studies, may indicate a beneficial effect of TSH-suppressive therapy as an immune-modulator in patients with differentiated thyroid carcinoma.

However, it must be highlighted that we did not measure the cytolytic activity of NK cells, yet in agreement with a possible stimulatory effect of TSH-suppressive therapy on NK cells expansion, others have reported that intraperitoneal injections of thyroxine increased NK cell counts in the spleen and bone marrow of adult mice (20), and that thyroxine increased IFN-induced cytotoxicity when administered simultaneously with IFN and IL-2 *in vitro* (21–23).

Of note, our present results strongly suggest that these immuno-modulatory effects are directly caused by thyroid hormones, considering that the patients studied here had no evidence of residual or recurrent thyroid cancer, and did not have any underlying autoimmune disorder.

On the contrary, most previous studies evaluating the effects of thyroid hormones on the immune system have been hampered by the inclusion of patients with autoimmune thyroid disease, or undergoing treatment with thionamides, factors that might have influenced the lymphoid populations and cytokine patterns encountered (4, 24–27). In this regard, increased serum IL-18 levels have been described in patients with Graves' disease, but not after liothyronine treatment in euthyroid individuals (28). To our knowledge, we describe here for the first time a thyroxine-dependent increase in serum IL-18 levels in an *in vivo* human model in which autoimmunity is not involved.

Graves' disease has also been associated with increased levels of IL-6 (29) and IL-12 (30), suggesting a strong and systemic stimulation of the immune response in autoimmune thyroid disease. On the other hand, no association of serum IL-6 concentrations with changes in thyroid function has been found (31, 32), in agreement with our present results. In our study, serum IL-6 levels remained unchanged despite marked changes in thyroid hormone levels, suggesting that the previously increased serum IL-6 levels reported in patients with Graves' disease (29) resulted from the autoimmune process underlying this disorder. In fact, it is well known that non-autoimmune thyroid disorders produce less striking changes on the immune response (33–35). In addition, we found that serum IL-12 and IFN-γ levels remained undetectable in controls and in patients throughout the study, possibly reflecting the predominantly local effects of these cytokines (9, 11).

Previous studies by Papic and colleagues (24, 36) suggested that thyroid hormone excess was associated
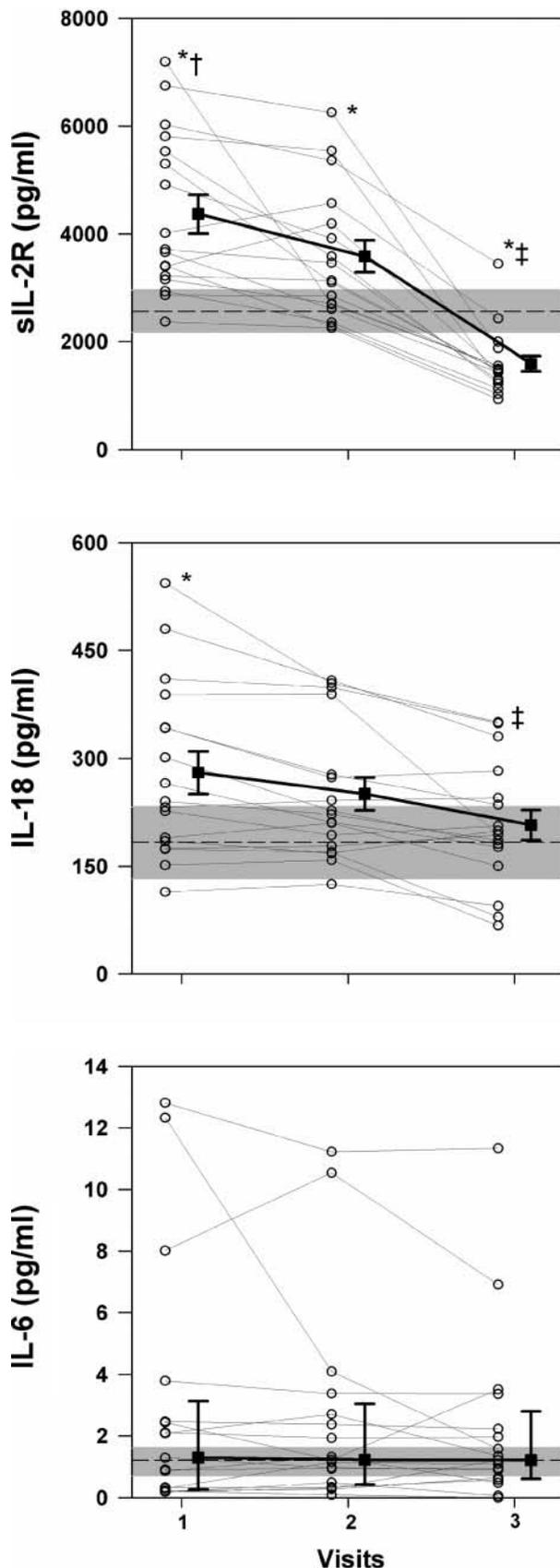
with suppression of peripheral blood NK cell activity, both in mice and in humans. In one of these studies, including 22 patients with Graves' disease and 18 hyperthyroxinaemic patients, a significant reduction in the cytolytic activity of NK cells against tumour cells was found, although the NK cell count in peripheral blood was not altered (24). However, only 3 patients with differentiated thyroid carcinoma were included in this study (24) and therefore the possibility exists that the finding of reduced NK cell-cytolytic activity was related to the immune milieu underlying Graves' disease rather than being directly dependent on thyroid hormone excess (37).

In our study, the percentage of CD4 + T lymphocytes in peripheral blood was diminished in patients when on TSH-suppressive thyroxine treatment. This finding might be related to previous treatment with ^{131}I, which might influence lymphoid populations, yet diagnostic or therapeutic doses of ^{131}I do not usually result in clinically relevant immuno-suppression (38). However, a transient decrease in NK cells, B and CD4 + T lymphocyte counts, occurring up to 15, 30 and 60 days respectively, has been reported after therapeutic doses of ^{131}I in patients with differentiated thyroid carcinoma (38). Nevertheless, our patients had not received ^{131}I for at least 6 months before recruitment.

Therefore, instead of being related to ^{131}I treatment, it is possible that the decrease in CD4 + T lymphocyte percentage observed in our patients during TSH-suppressive thyroxine treatment may reflect a response similar to that observed in patients with renal carcinoma after treatment with IFNα-2b and IL-2, consisting of an increase in the cytolytic activity of NK cells paralleled by a significant decrease in the percentage of CD4 + lymphocytes, with no changes in the proliferative response to T-cell mitogenic signals (39).

In conclusion, our present study shows that thyroid hormones exert important direct and reversible stimulatory effects on serum markers of cell-mediated immune response, consisting of an increase in serum IL-18 and sIL-2R levels with a concomitant expansion of NK cells in peripheral blood. Whether these effects contribute to the good overall prognosis of patients with differentiated thyroid carcinoma on TSH-suppressive therapy remains to be established.

Figure 2 Serum cytokine levels in patients with differentiated thyroid carcinoma on suppressive thyroxine treatment and during thyroxine withdrawal, and also compared with euthyroid controls. Data are represented as means±S.E.M. for sIL-2R and IL-18, and median ± interquartile range for IL-16 (these data did not follow a normal distribution), and the dot scattergram shows the individual data with lines connecting them. The shaded areas represent the 95% confidence interval of the mean (dashed line) observed in the control group for sIL-2R and IL-18, and the 95% confidence interval of the median (dashed line) for IL-6. *$P < 0.05$ or less compared with euthyroid controls; †$P < 0.05$ or less compared with visit 2; ‡$P < 0.05$ or less compared with both visits 1 and 2.

Acknowledgements

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